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VIRULENCE FACTORS IN CLINICAL ISOLATES OF *ENTEROCOCCUS*

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ABSTRACT

Enterococci have been recognised as being potentially pathogenic for humans since the early 1900s, when they were well establish as a cause of endocarditis and urinary tract infections. This observation strongly supports the existence of additional virulence properties that may facilitate or enhance virulence of enterococci associated with infections. These include Cytolysin, aggregation substance, extracellular superoxide, surface carbohydrate and surface proteins such as *Esp*, Gelatinase. One hundred and twenty-five isolates of *Enterococcus* species from various clinical specimens were evaluated for the presence of virulence determinants like hemolysin production, gelatinase production and biofilm formation by phenotypic tests. A total of 125 *Enterococcus* isolates, 84 are *Enterococcus faecalis* and 41 are *Enterococcus faecium.* Of the 84 species of *Enterococcus faecalis* tested for the production of haemolysin, 43.2% shows beta haemolysis on blood agar, 10.7% shows gelatinase production and 33.3 % shows biofilm production. In case of *Enterococcus faecium* 43.6 shows beta haemolysis, 14.6% shows gelatinase and 39% shows biofilm production. We conclude the virulence determinants have been widely prevalent in enterococcal isolates from clinical origin.

Key Words: *Enterococcus*, Virulence factors, Haemolysin, Gelatinase, Biofilm.

INTRODUCTION:

Enterococcus was first described in 1899 as a new *Streptococcus* of enteric origin. The likely intestinal source of a similar gram positive coccus recovered from a patient with endocarditis in 1906 determined the choice of name "*Streptococcus faecalis*" [1]. In the mid 1930s streptococcus were classified into four divisions: pyogenic, viridians, lactic and enterococcus based on biochemical and physiological properties [2]. This observation strongly supports the existence of additional virulence properties that may facilitate or enhance virulence of enterococci associated with infections [3]. Several potential virulence factors have been identified in enterococci in recent years and these have been found primarily in *E. faecalis*. These include Cytolysin, aggregation substance, extracellular superoxide, surface

carbohydrate and surface proteins such as *Esp*, Gelatinase [4]. This study was carried out to study the elaboration of virulence factors by strains of *Enterococcus* isolated from clinical specimens.

MATERIAL AND METHODS:

The study was conducted in the Department of Microbiology, National Institute of Medical Sciences & Research, Jaipur, from March 2018 to February 2019. A total of 125 clinical isolates of *Enterococcus* species were included in this study. The samples from which these isolates were obtained included sterile body fluids like blood, cerebrospinal fluid (CSF), peritoneal fluid, pleural fluid, isolates from urine samples and pus. Routine bacteriological methods were followed for the isolation and identification of *Enterococcus* species [5-7]. The study was approved by the institutional ethics committee.

A. **Hemolysin detection:** Production of hemolysin was determined by plating the enterococcal isolates onto Brain Heart Infusion (BHI) agar supplemented with 5% human blood. Plates were incubated at 37°C and observed after 24 and 48 hours. A clear zone of β hemolysis around the bacterial colonies indicated the production of hemolysin. All strains inoculated onto human blood agar were also inoculated onto agar containing 5% sheep blood. This was to look for strains of *Enterococcus*, which show β hemolytic colonies on both sheep blood agar and human blood agar [8].
B. **Detection of Gelatinase production:** *Enterococcus* isolates were inoculated onto peptone yeast extract agar containing gelatin (30 g/L) and incubated at 37°C for 24 hours. The plates with growth were then cooled to ambient temperature for two hours. Appearance of a turbid halo or zone around the colonies was considered to be an indication of gelatinase production [8].

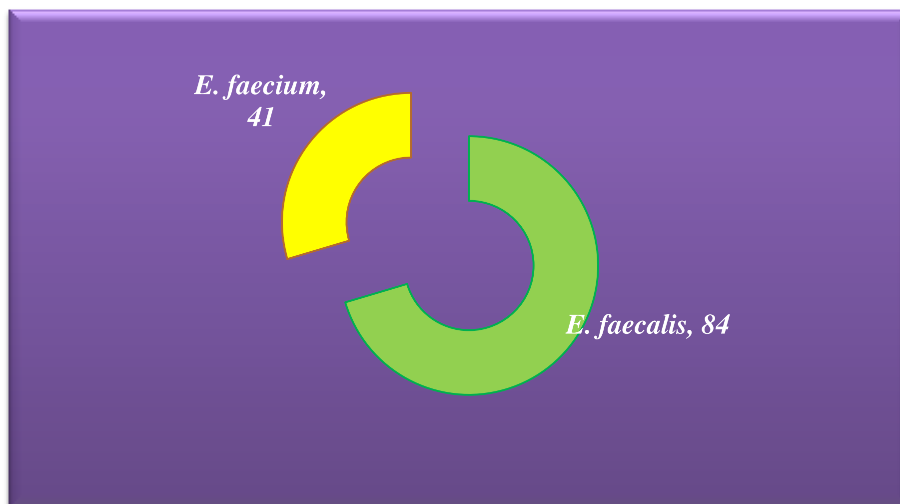
C. **Bio-film production by Microtitre plate method:** This procedure was performed as described earlier with a few modifications. Colonies of enterococci, which had grown overnight on blood agar were inoculated in Trypticase Soy Broth (TSB) (Hi-media laboratories, Mumbai, India)[9] with 2% sucrose and incubated at 37°C overnight. This overnight growth was diluted 1: 100 in the TSB with sucrose. 200µL of these diluted inoculums was added onto sterile flat-bottomed polystyrene microtiter plates. The microtiter plates were incubated aerobically at 37°C for 48 hours. At the end of 48 hours, the culture was discarded from the wells. The wells were gently washed with PBS (pH 7.2) to remove non-adherent planktonic cells. Manual washing was done using a multichannel micropipette. The adherent biofilm were then fixed by using 2% sodium acetate for 20 minutes. The plates were then dried at room temperature and finally stained with 0.1% safranin for 20 minutes. The plates were then washed five times as previously described and dried. The absorbance of the biofilm on the bottom surface of each well was determined at 490 nm with an ELISA micro plate reader. All strains were inoculated in triplicate on the microtiter plate and all experiments were repeated three times. For calculating the absorbance, the average of the absorbance for each of the three wells inoculated with a single strain was

calculated. All experiments also included three blank wells (i.e., culture medium without any bacteria). At 490 nm, biofilm formation was considered to be high when the absorbance was > 0.2 OD, moderate when it was between 0.20 and 0.10 and weak/absent when < 0.10 [8].

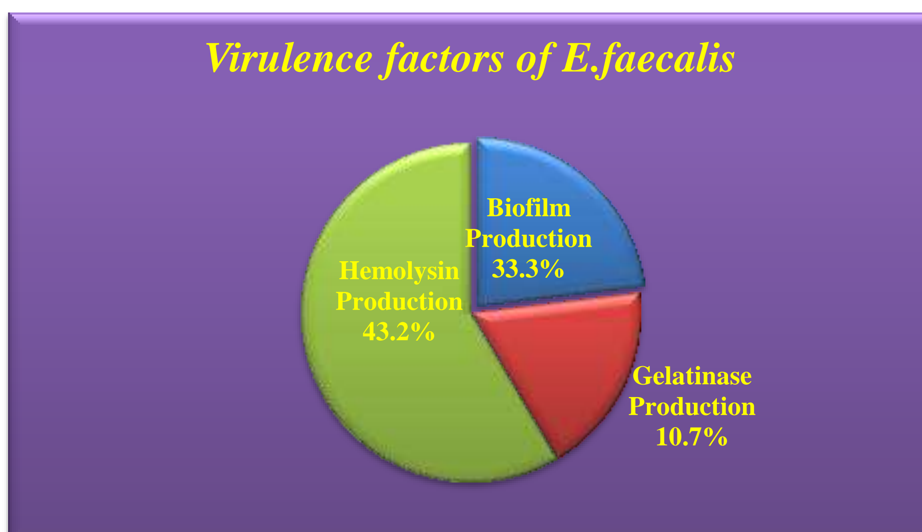
RESULTS:

A total of 125 *Enterococcus* isolates, 84 are enterococcus faecalis and 41 are enterococcus faecium. Of the 84 species of enterococcus faecalis tested for the production of haemolysin, 43.2% shows beta haemolysis on blood agar, 10.7% shows gelatinase production and 33.3 % shows biofilm production. In case of enterococcus faecium 43.6 shows beta haemolysis, 14.6% shows gelatinase and 39% shows biofilm production.

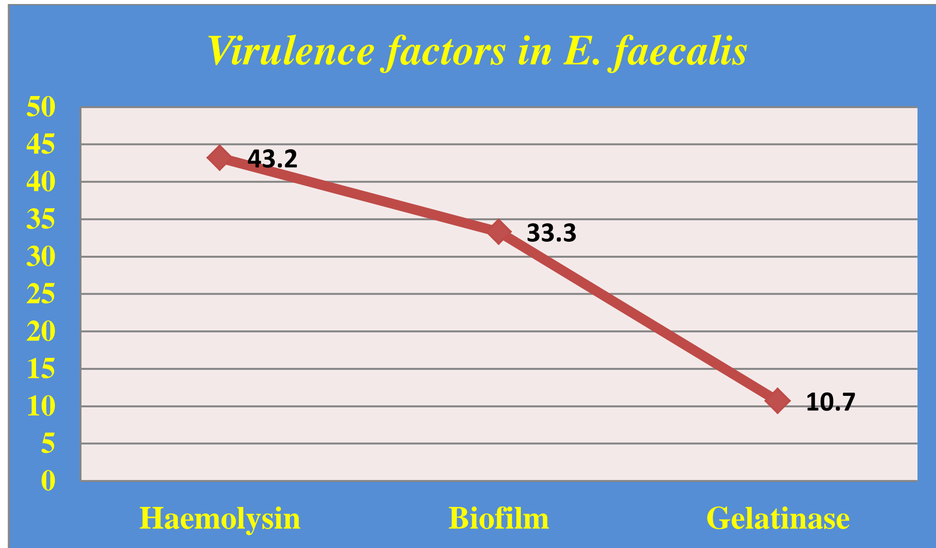
Prevalence of *Enterococcus faecalis* and *Enterococcus faecium*



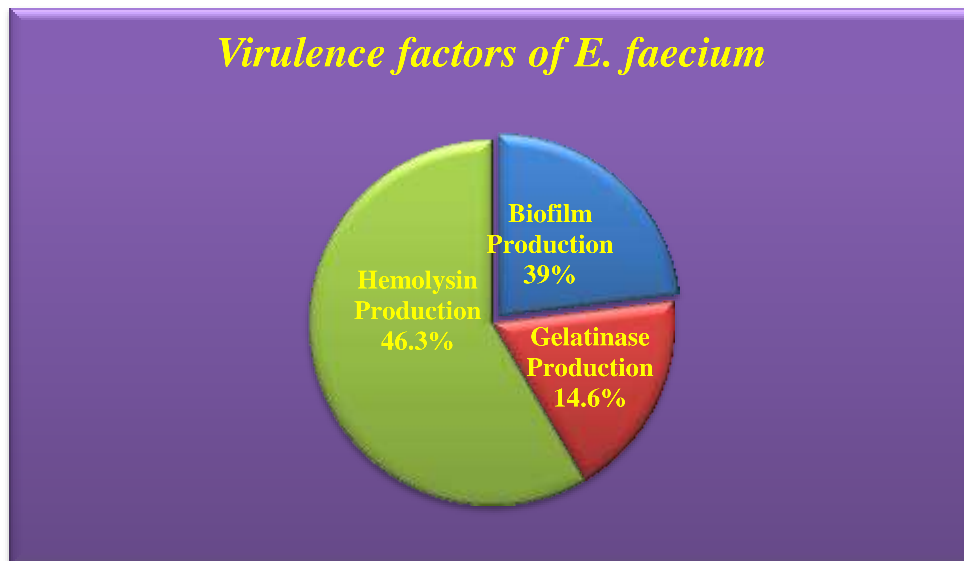
Virulence factors in clinical isolates of *Enterococcus faecalis (n-84).*



Virulence factors in clinical isolates of *Enterococcus faecalis (n-84).*



Virulence factors in clinical isolates of *Enterococcus faecium (n-41).*



Virulence factors in clinical isolates of *Enterococcus faecium (n-41).*

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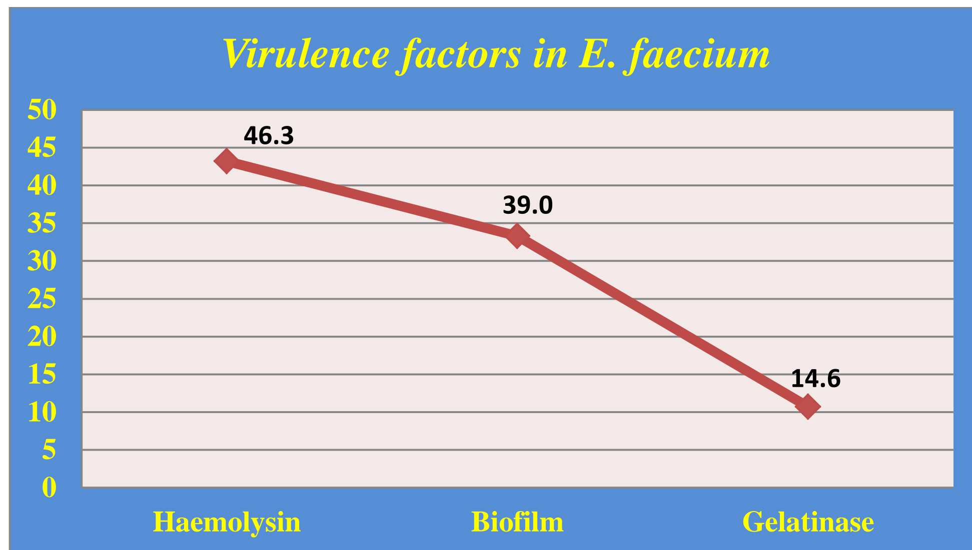

DISCUSSION:

In our study the expression of three virulence factors was evaluated – hemolysin, gelatinase and biofilm formation. Adherence to body surface is considered a major factor responsible for the clinical isolates of enterococcus. Strains causing infections are considered to have a great capacity to adhere to surface than commensal strains [10]. Various studies on virulence factors of enterococci have currently reported their widespread distribution [11]. As compared our study with recent study from south India, hemolysin production was seen in 82% of clinical isolates, while gelatinase production was demonstrated 40.6% of the isolate [12-13]. In another study from south India in 2013 reported that the hemolysin production in *Eneterococcus* isolates was 21%, gelatinase production was reported about 19% in total isolates and biofilm production in enterococcus isolates was reported 53% of total [14]. In our study we also reported the hemolysin production in enterococcus faecalis 43.2%, and e. faecium was 46.3% and gelatinase production in enterococcus faecalis 10.7%, in e. faecium 14.6 and biofilm production was reported in our study in e. faecalis 33.3%, in e. faecium 39.0%. *Enterococci* have been associated with biofilm on various kind of indwelling devices like prosthetic heart valves, urinary catheter etc. and this capability to produce biofilm has been consider an important virulence factor of these organisms[15]. Various methods like microscopic biofilm formation assay and epifluorescence microscopic have been used by many authors to study the biofilm forming capacity of bacteria [16]. However, the method that has been used frequently in recent times is the Microtitre plate biofilm production assay [17]. This method is preferred because of its simplicity and cost-effectiveness. The Microtitre plate biofilm assay technique was first devised for studying the biofilm capability of *Listeria monocytogenes* by Djordjevic D, Wiedmann M et al. in 2002 [18]. This method later modified and used for the studying biofilm formation in other gram positive bacteria like coagulase negative staphylococcus and enterococcus species. Hemolysin production and gelatinase production was seen in 24% and 19.2% in all clinical isolates reported by some studies [14]. Gelatinase elaborated by some enterococcus isolates has been identified to be an extracellular zinc-endopeptides capable of hydrolyzing gelatin, collagen, casein, haemoglobin and other

peptides [19]. The role of gelatinase in causing endocarditis has been studied using animal models [20]. The role of gelatinase in enterococcal infection is providing nutrition to the bacteria by degradation of host tissue [21].

CONCLUSION:

Enterococcus infections are one of the most important global health problems causing considerable morbidity in the general population. We studied the two major species associated with most of the enterococcal infections, namely *E. faecalis* and *E. faecium.* Whereas, *E. faecalis* is the commonly reported species, *E. faecium* is equally gaining the importance and is the major isolated species in many of the studies. In our study the expression of three virulence factors was evaluated – hemolysin, gelatinase and biofilm formation. Although a majority of the clinical isolates of enterococci tested were found to be positive for hemolysin production in our study, contrary to some other Indian studies. Adherence to body surface is considered a major factor responsible for the pathogenicity of the clinical isolates of enterococcus. Strains causing infections are considered to have a great capacity to adhere to surface than commensal strains.

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